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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,407,755**, on October 11, 2002, by **THE HOSPITAL FOR SICK CHILDREN**, assignee of Chaim M. Roifman, Amos J. Simon, Peter M. Demin and Olga B. Rounova, for "Inhibition of VEGF Secretion".

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INHIBITION OF VEGF SECRETION

FIELD OF THE INVENTION

[0001] This invention relates to therapeutic organic compounds and inhibition of secretion of vascular endothelial growth factor (VEGF) and its effects, including angiogenesis.

BACKGROUND OF THE INVENTION

[0002] VEGF is a disulfide-linked, dimeric glycoprotein with multifunctional properties. There are at least 5 isoforms derived from alternative splicing of a single gene, encoding proteins of 121, 145, 165, 189, and 206 amino acid residues. The isoforms differ in their degree of heparin-binding and sequestration in the extracellular matrix (ECM). VEGF (also known as VEGF-A) belongs to a family of growth factors that includes placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E.

[0003] VEGF is potent stimulator of angiogenesis, the formation of new capillaries from pre-existing blood vessels. VEGF is an endothelial cell-specific mitogen and, further, VEGF induces non-proliferative endothelial cell activities involved in the angiogenic process, cell migration and invasion. VEGF induces secretion of proteolytic enzymes which degrade the basement membrane and extracellular matrix, nitric oxide release, expression of adhesion molecules, and cell morphological changes. VEGF acts as a survival factor for endothelial cells by inducing the expression of anti-apoptotic proteins.

[0004] VEGF is also a potent stimulator of vascular permeability. Discovered separately as a vascular permeability factor (VPF), VEGF causes vascular leakage with a potency 50 000 times that of histamine. Excessive vascular permeability may contribute to angiogenesis by enhancing protein extravasation, or it may represent a distinct factor in disease processes by leading to edema and swelling.

[0005] VEGF exerts its effects primarily via two endothelial receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). VEGFR-1 and -2 are thought

to mediate different signal transduction functions. VEGFR-1 binds VEGF, VEGF-B, and PlGF; VEGFR-2 binds VEGF, VEGF-C, VEGF-D, and VEGF-E. VEGFR-1 shows higher affinity to VEGF. It is hypothesized that VEGFR-2, which is expressed almost exclusively in proliferating vessels, is responsible for driving endothelial cell angiogenic processes. VEGFR-1, which is expressed in hematopoietic stem cells and inflammatory cells such as monocytes and macrophages in addition to endothelial cells, is believed to mediate the activation and recruitment of these cells in response to VEGF and PlGF.

[0006] Through its angiogenic, permeability-enhancing and inflammatory effects, VEGF has been implicated in the pathophysiology of several diseases, including cancers, ocular diseases, rheumatoid arthritis, endometriosis, psoriasis, and atherosclerosis.

[0007] The critical role of tumour angiogenesis in the progression of cancer is well known. Neovascularization is necessary for the development of solid tumours beyond a few cubic millimetres in size as new blood vessels are required to provide oxygen and nutrients to the tumour cells; the new blood supply also allows tumour cells to enter the circulation and metastasize. VEGF expression is markedly upregulated in tumours and VEGF receptors are upregulated on the tumour endothelium. Numerous studies have demonstrated a correlation between tumour vascularity and metastasis or patient prognosis. In addition to solid tumours, angiogenesis has been reported to play a role in hematological disorders such as leukemias, lymphomas and myeloproliferative disorders, in which there is increased bone marrow vascularity and elevated levels of angiogenic factors. A role for VEGF in the development of ascites and pleural effusion, which cause difficult symptoms for advanced-stage cancer patients, has also been postulated.

[0008] Ischemic retinopathies, such as diabetic retinopathy and retinopathy of prematurity, are leading causes of blindness in adults and children, respectively, in the developed world. These diseases are marked by retinal neovascularization. Increased levels of VEGF are found in the vitreous and retina of patients with diabetic retinopathy. VEGF is also increased in diabetic retinal tissue without overt

retinopathy and so may also play a role in the early development of the disease by mediating breakdown of the blood-retinal barrier and increased retinal vascular permeability, leading to vessel leakage and macular edema. In other eye conditions such as age-related macular degeneration, a significant cause of vision loss in aging populations, choroidal neovascularization occurs which has been shown to involve overexpression of VEGF by retinal pigment epithelial cells and choroidal fibroblasts.

[0009] In rheumatoid arthritis, an increased vascular supply to the synovium is thought to underlie the expansion of the synovial lining of joints and the development of joint destruction. Angiogenesis is involved in the formation and maintenance of the highly vascularized pannus. Increased serum VEGF levels in patients is associated with disease activity. VEGF expression in rheumatoid synovial cells such as fibroblasts or activated leukocytes is involved in the angiogenic process in rheumatoid arthritis, and may also act as a vascular permeability factor, thus increasing edema and joint swelling.

[0010] Endometriosis is characterized by significant vascularization within and surrounding ectopic endometrial tissue. A new blood supply is thought to be essential for the survival of the ectopic endometrial implant and the development of the disease. There are elevated levels of VEGF in the peritoneal fluid of women with endometriosis, secreted by peritoneal fluid and ectopic tissue macrophages.

[0011] Upregulation of VEGF and VEGF receptors occurs in psoriasis and other skin diseases. Increased circulating VEGF, seen in increased plasma VEGF levels, may cause systemic vascular hyperpermeability. Upregulated VEGF and VEGF receptors in the blister fluids and the lesional epidermis of patients with bullous diseases (bullous pemphigoid, erythema multiforme and dermatitis herptiformis) suggests that VEGF is a factor in the development hyperpermeable dermal microvessels and papillary edema.

[0012] A possible role for VEGF has been proposed in atherosclerosis. Neovascularization occurs in atherosclerotic plaques and may be required for plaque progression. Raised levels of VEGF, which are expressed by neovascular endothelial cells, smooth muscle cells and inflammatory cells, are found in patients with arterial

disease. In animal models, VEGF has been shown to increase atherosclerotic plaque formation and the numbers of macrophages, while inhibition of VEGFR-1 has been described as reducing atherosclerotic plaque growth through inhibition of inflammatory cell infiltration.

[0013] Accordingly, VEGF inhibitors may provide an effective treatment for these diseases and other diseases related to VEGF.

SUMMARY OF THE INVENTION

[0014] The present invention is based on the unexpected discovery that the compounds (E,E)-2-(benzylaminocarbonyl)-3-(3,4-dihydroxystyryl)acrylonitrile(CR4), (E,E)-2-(3,4-dihydroxybenzylaminocarbonyl)-3-(3,5-dimethoxy-4-hydroxystyryl)acrylonitrile (CR11), and (E,E)-2-(3,4 dihydroxybenzylaminocarbonyl)-3-styrylacrylonitrile (CR19) are capable of inhibiting VEGF secretion. These compounds are described in co-pending US application 09/834,728, the contents of which are hereby incorporated by reference.

[0015] In its broadest aspect, the invention relates to inhibition of secretion of VEGF.

[0016] In one aspect, the invention provides a method of inhibiting secretion of VEGF, in an animal in need of such inhibition, comprising administering to the animal an effective amount of CR4, CR11 or CR19.

[0017] In another aspect, the invention provides a method of inhibiting effects of VEGF, including angiogenesis, and a method of treating a disorder related to VEGF in an animal in need of such inhibition or treatment. In various embodiments the animal is a human patient and the disorder is cancer, rheumatoid arthritis, retinopathy and atherosclerosis.

[0018] In another aspect, the invention relates to use of CR4, CR11 or CR19 to inhibit VEGF secretion or effects of VEGF or to treat a disorder related to VEGF. The invention also relates to use of CR4, CR11 or CR19 to prepare a medicament to inhibit VEGF secretion, or effects of VEGF or to treat a disorder related to VEGF.

[0019] Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1 is a graph showing inhibition of VEGF secretion from HTB-133 (KDR+) and HTB-131 (KDR-) breast cancer cell lines by CR-4.

[0021] Figure 2 is a graph showing inhibition of VEGF secretion from breast cancer MDA-231 cell line by CR4.

[0022] Figure 3 is a graph showing inhibition of VEGF secretion from HTB-72 melanoma cell line by CR4.

[0023] Figure 4 is a graph showing inhibition of VEGF secretion from CR2-1730 HUVEC cell line by CR4.

[0024] Figure 5 is a graph showing inhibition of HUVEC growth by CR-4 induced VEGF depleted media of HTB-133 (KDR+) and HTB-131 (KDR-) breast cancer cell lines and rescue by recombinant human VEGF (10 ng/ml).

[0025] Figure 6 is a graph showing inhibition of VEGF secretion from breast cancer cell lines HTB-133 and MDA-231 by CR11.

[0026] Figure 7 is a graph showing inhibition of VEGF secretion from breast cancer cell lines HTB-133 and MDA-231 by CR19.

DETAILED DESCRPTION OF THE INVENTION

[0027] The inventors have discovered that CR4, CR11 and CR19 inhibit secretion of VEGF. These compounds inhibit, in a dose dependent manner, VEGF secretion

from breast cancer cell lines, melanoma cell line and human umblilical vascular endothelial cell line. The IC50 values for all three compounds were about 30 nM, suggesting a common target with high affinity for these compounds. CR4, CR11 and CR19 therefore provide a new therapeutic approach that target expression and secretion of VEGF.

[0028] The compounds, by inhibiting VEGF secretion, also inhibit the effects of VEGF. VEGF, first identified as an endothelial cell specific growth factor, stimulates endothelial cell proliferation. CR4, by inhibiting secretion of VEGF, was found to inhibit endothelial cell proliferation. In particular, when CR4 is added to breast cancer cell lines which normally secrete VEGF, the secretion of VEGF to the medium is inhibited and the medium is unable to stimulate proliferation of human umbilical vascular endothelial primary cells (these cells do not secrete detectable amount of VEGF). The inhibition of endothelial cell growth is however rescued if recombinant VEGF is added to the medium, confirming that inhibition results from inhibition of VEGF secretion. The inhibition of cell growth is also CR4 dose dependent, with an IC50 value of about 20 –30 nM. The effective dose is therefore similar to that required for inhibition of VEGF secretion and further confirms that inhibition of cell growth results from inhibition of VEGF secretion.

[0029] These results indicate that CR4, CR11 and CR19 therefore can be used to inhibit the effects of VEGF. Furthermore, while VEGF is an essential part of normal embryonic development, repair, tissue regeneration, female reproductive cycle and other physiological processes, the effects of VEGF is associated with variety of disorders. In many of these disorders, VEGF expression/levels are upregulated. The compounds therefore may also be used to treat these disorders. By way of an example, VEGF plays a central role in angiogenesis (and vasculogenesis during embryonic development), the formation of new blood vessels, and it is known that angiogenesis is critical to growth and metastasis of tumour cells in the progression of cancer. The compounds therefore may be used to treat cancer, including, by inhibiting tumour growth.

[0030] The invention therefore provides a method of inhibiting VEGF secretion

comprising administering to an animal in need of such inhibition an effective amount of CR4, CR11, or CR19. The invention also provides a method of inhibiting effects of VEGF in an animal in need of such inhibition. The term "effects of VEGF" is used to refer to effects resulting from, or mediated by VEGF activity, including via its receptor(s), and include angiogenesis, vasculogenesis, arteriogenesis, vascular permeability, inflammation. The term inhibition or inhibiting or like terms are used to broadly refer to any reduction of targetted charateristic which is statistically significant when compared to a control (ie no administration of the compounds) as can be measured using techniques known in the art.

[0031] The animal may be a human patient suffering from disorders related to VEGF. It is meant by such disorders any disorder in which the VEGF is believed to play a role in the progression or symptoms of the disorder or in which VEGF expression and/or levels are upregulated. Disorders related to VEGF include: cancer, including solid tumour cancers such as breast , pancreatic, colon and brain cancer, melanoma; diabetes; retinopathy, including diabetic retinopathy; corneal conjunctival vascularization; hemangioma; Karposi's sarcoma; atherosclerosis. The disorders also include rheumatoid arthritis, atherosclerosis, endometriosis, psoriasis, hematological malignancy, including leukemia, lymphoprokuferative disorder or myeloproliferative disorder, renal vein occlusion, retinopathy of prematurity, age-related macular degeneration, bullous diseases.

[0032] In another embodiment, the invention provides a method of treating a disorder related to VEGF comprising administering an effective amount of CR4, CR11, or CR19 to an animal in need of such treatment. In one embodiment, the animal is a human patient and the disorder is cancer, including tumourous cancer. In a specific embodiment, the growh of tumour is inhibited.

[0033] The compounds may be used in the form of the free base, or in other forms such as salts, prodrugs, solvates, and hydrates, and reference to CR4, CR11 and CR19 are intended to encompass all such forms of the compound. The acids which can be used to prepare acid addition salts are those which produce, when combined with the compound, pharmaceutically acceptable salts, that is, salts whose anions are non-toxic

to the animal in pharmaceutical doses of the salts, so that the beneficial properties inherent in the free base are not vitiated by side effects ascribable to the anions. Pharmaceutically acceptable salts include those derived from the following acids; mineral acids such as hydrochloric acid, sulfuric acid, phosphoric acid and sulfamic acid; and organic acids such as acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, cyclohexysulfamic acid, quinic acid, and the like.

[0034] Similarly, basic addition salt may be prepared using an inorganic base such as lithium, sodium, potassium, calcium, magnesium or barium hydroxide. Illustrative organic bases which form suitable salts include aliphatic, alicyclic or aromatic organic amines such as methylamine, trimethylamine and picoline or ammonia.

[0035] The selection of other pharmaceutically acceptable salts will be known to a person skilled in the art and a desired salt may be prepared using standard techniques.

[0036] Prodrugs of the compounds may be conventional esters formed with available hydroxy, amino or carboxyl group on the compound. For example, an OH group may be acylated using an activated acid in the presence of a base, and optionally, in inert solvent (e.g. and acid chloride in pyridine). Some common esters which have been utilized as prodrugs are phenyl esters, aliphatic (C_8-C_{24}) esters, acyloxymethyl esters, carbamates and amino acid esters. Conventional procedures for the selection and preparation of suitable prodrugs are described, for example, in "Design of Prodrugs" ed. H. Bundagaard, Elsevier, 1985.

[0037] A "solvate" is formed when a suitable solvent are incorporated in the crystal lattice of the compound or salt thereof. A suitable solvent is physiologically tolerable at the dosage administered. Examples of suitable solvents are ethanol, water and the like. When water is the solvent, the molecule is referred to as a "hydrate". Methods to prepare a solvate are known in the art. In general, solvates are prepared by dissolving the compound in the appropriate solvent and isolating the solvate by cooling or using an antisolvent. The solvent is typically dried or azeotroped under ambient conditions.

[0038] The compounds may be administered alone in combination with a pharmaceutically acceptable carrier, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice.

[0039] Preferably the compounds are formulated into pharmaceutical compositions in a biologically compatible form suitable for administration *in vivo*. Accordingly, in one embodiment, CR4, CR11 or CR19 are administered to a human patient in combination with a pharmaceutically acceptable carrier.

[0040] The compositions containing the compounds can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffer solutions with a suitable pH and iso-osmotic with the physiological fluids.

[0041] The compounds may be administered to a patient in a variety of forms depending on the selected route of administration, as will be understood by those skilled in the art. The compositions of the invention may be administered orally or parenterally. Parenteral administration includes intravenous, intraperitoneal, subcutaneous, intramuscular, transepithelial, nasal, intrapulmonary, intrathecal, rectal and topical modes of administration. Parenteral administration may be by continuous infusion over a selected period of time.

[0042] The compounds may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the compound of the invention may be incorporated with excipient and used in the form of ingestible

tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers and the like.

[0043] The compounds may also be administered parenterally or intraperitoneally. Solutions of a compound can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, DMSO and mixtures thereof with or without alcohol, and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. A person skilled in the art would know how to prepare suitable formulations. Conventional procedures and ingredients for the selection and preparation of suitable formulations are described, for example, in Remington's Pharmaceutical Sciences (1990 - 18th edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999.

[0044] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersion and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists.

[0045] An effective amount of the compounds refers to the amount sufficient to inhibit secretion or effects of VEGF, or in the case of treatment of a disorder related to VEGF, the amount sufficient to alleviate, improve, mitigate, ameliorate or cure the disorder or one or more symptoms of the disorder. The clinical effects resulting from inhibition of VEGF secretion or effects of VEGF or treatment of a disorder related to VEGF may be assessed in the known manner, for example, in the case of effect on tumour growth, by tumour shrinkage. The effective amount can vary depending on many factors such as the pharmacodynamic properties of the compound, the mode of administration, the age, health and weight of the recipient, the nature and extent of the symptoms, the frequency of the treatment and the type of concurrent treatment, if any, and the clearance rate of the compound in the animal to be treated. One of skill in the art can determine the appropriate dosage based on the above factors. The compounds may be administered initially in a suitable dosage that may be adjusted as required,

depending on the clinical response.

[0046] The compounds may be packaged as a kit and the invention in one aspect provides a kit comprising CR4, CR11 or CR19 and instructions for use of the compound, including to inhibit secretion or effects of VEGF or to treat a disorder related to VEGF.

[0047] All references cited herein are fully incorporated by reference.

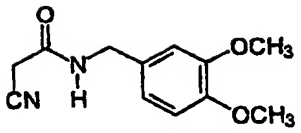
Examples

[0048] The compounds CR4, CR11 and CR19 may be prepared as described in Examples 1 to 14.

[0049] *Materials and Methods for Examples 1-14*

[0050] ^{1}H NMR spectra were obtained on a Varian Unity Plus spectrometer (USA) at 500 MHz with tetramethylsilane (TMS, Me_4Si) as an internal standard (δ=0). Electrospray mass spectra were recorded on an API III Plus triple quadrupole mass spectrometer (USA), with a direct introduction of the samples into the ionization source. Thin layer chromatography was performed with UV-254 aluminum-backed TLC sheets of 0.25 mm thickness (Kieselgel 60 F_{254}, Merck, Germany). HPLC separation of the compound of Example 13 was performed on a Waters 600 chromatograph (USA), column Nova-Pak C18 3.9 × 300 mm (Waters, USA). Vacuum distillations were done using Kugelrohr apparatus (Aldrich, USA) at stated temperatures of an oven. 3,5-Dimethoxy-4-hydroxycinnamaldehyde, 3,4-dimethoxycinnamic acid, 3,4-dihydroxycinnamic acid, 3,4-dimethoxybenzylamine, benzylamine, methyl cyanoacetate, were purchased from Aldrich (USA) and were used as received. The reagents were from Aldrich (USA). Solvents were purchased from Caledon (Canada).

[0051] Example 1: N-(Cyanoacetyl)3,4-dimethoxybenzylamide (A_1)



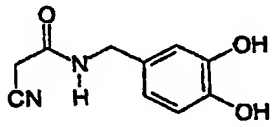
[0052] To 3,4-dimethoxybenzylamine (2.7 ml, 18 mmol) methyl cyanoacetate was added (1.6 ml, 18 mmol). The reaction was heated for 14 h at 100°C. Cooling gave a dark brown solid which was recrystallized from ethanol to give 2.90 g of the product (69% yield).

[0053] The product gave the following analytical data:

[0054] NMR (CD_3COCD_3, δ, ppm): 3.62 (s, 2H, CH_2CN), 3.78 (s, 6H, $(OMe)_2$), 4.34 (br.s., 2H, $NHCH_2Ph$), 6.84 (dd, 1H, J 1.95 and 8.1 Hz, H^6), 6.88 (d, 1H, J 8.1 Hz, H^5), 6.93 (d, 1H, J 1.95 Hz, H^2), 7.80 (br.s., 1H, NH).

[0055] MS, m/e (rel. intensity, %): 235 (19) $[M+H]^+$, 252 (100) $[M+NH_4]^+$, 257 (33) $[M+Na]^+$.

[0056] Example 2: N-(Cyanoacetyl)3,4-dihydroxybenzylamide (A_2)



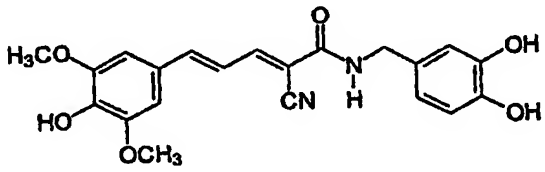
[0057] To N-(cyanoacetyl)3,4-dimethoxybenzylamide (Example 1, 0.2 g, 0.85 mmol) in 20 ml of CH_2Cl_2 boron tribromide was added under argon at -78°C (0.24 ml, 2.56 mmol) in 2.5 ml of CH_2Cl_2. After 2 h the reaction was brought to room temperature and stirred overnight. The reaction was cooled to 0°C, 10 ml of 1N HCl was added, the solution was extracted with 3 × 50 ml of ethyl acetate, the organic phase was washed to neutral pH, dried with $MgSO_4$, and taken to dryness. The residue was purified by silica gel chromatography ($CHCl_3$-MeOH, 20:1) to give a yellow solid (0.07 g, 40% yield). The product gave the following analytical data:

[0058] NMR (CD_3COCD_3, δ, ppm): 2.83 (s, $(OH)_2$), 3.60 (s, 2H, CH_2CN), 4.25

(br.s., 2H, $NHCH_2Ph$), 6.63 (dd, 1H, J 1.95 and 8.1 Hz, H^6), 6.75 (d, 1H, J 8.1 Hz, H^5), 6.79 (d, 1H, J 1.95 Hz, H^2), 7.71 (br.s., 1H, NH).

[0059] MS, m/e (rel. intensity, %): 207 (38) $[M+H]^+$, 224 (100) $[M+NH_4]^+$, 229 (2.6) $[M+Na]^+$.

[0060] Example 3: (E,E)-2-(3,4-Dihydroxybenzylaminocarbonyl)-3-(3,5-dimethoxy-4-hydroxystyryl)acrylonitrile (CR11)

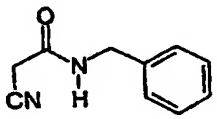


[0061] To 3,5-dimethoxy-4-hydroxycinnamaldehyde (0.042 g, 0.2 mmol) and N-(cyanoacetyl)3,4-dihydroxybenzylamide (Example 2, 0.042 g, 0.2 mmol) in 10 ml of ethanol 3 mg of β-alanine was added and the reaction was refluxed for 6 h. Water was added and the solid was recrystallized from 5 ml of ethanol twice to give 0.06 g (75%) of a red solid. The product gave the following analytical data:

[0062] NMR (CD_3COCD_3, δ, ppm): 2.81 (s, $(OH)_3$), 3.89 (s, 6H, $(OMe)_2$), 4.39 (br.s., 2H, $NHCH_2Ph$), 6.68 (dd, 1H, J 1.95 and 8.1 Hz, $H^{6'}$), 6.76 (d, 1H, J 8.1 Hz, $H^{5'}$), 6.86 (d, 1H, J 1.95 Hz, $H^{2'}$), 7.07 (br.s, 2H, H^{2+6}), 7.16 (dd, 1H, J 11.7 and 15.1 Hz, PhCCHCCN olefinic), 7.37 (d, 1H, J 15.1 Hz, PhCH olefinic), 7.70 (br.s., 1H, NH), 7.98 (dd, 1H, J 0.75 and 11.7 Hz, CHCN olefinic).

[0063] MS, m/e (rel. intensity, %): 397 (100) $[M+H]^+$, 414 (14) $[M+NH_4]^+$.

[0064] Example 4: N-(Cyanoacetyl)benzylamide (A_3)



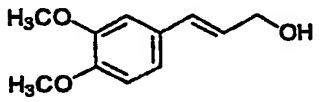
[0065] The compound was prepared as described in Example 1 by adding methyl

cyanoacetate (1.3 ml, 14 mmol) to benzylamine (1.5 ml, 14 mmol). The compound was distilled *in vacuo* directly from the reaction mixture (Kugelrohr apparatus (Aldrich), 0.1 mm Hg, T. oven 180-190°C) to give an off-white solid (2.34 g, 95%). The product gave the following analytical data:

[0066] NMR (CD_3COCD_3, δ, ppm): 3.39 (s, 2H, $CNCH_2$), 4.46 (d, 2H, J 5.4 Hz, $NHCH_2Ph$), 6.40 (br.s., 1H, NH), 7.24-7.36 (m, 5H, Ph).

[0067] MS, m/e (rel. intensity, %): 175 (64) $[M+H]^+$, 192 $[M+NH_4]^+$.

[0068] Example 5: 3,4-Dimethoxycinnamyl alcohol (A_6)

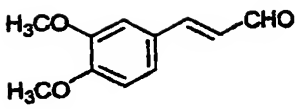


[0069] To a solution of 0.42 g (2.0 mmol) of 3,4-dimethoxycinnamic acid in 50 ml MeOH was added $SOCl_2$ (50 µl) and the mixture was stirred at 60°C for 5 h. Methanol was taken to dryness and the obtained 3,4-dimethoxycinnamic acid methyl ester was reduced with 1M THF solution of diisobutylaluminum hydride (8.0 mmol) in absolute THF (50 ml) at 20°C for 1 h. Water was added, the mixture was extracted with EtOAc, dried with $MgSO_4$ and distilled *in vacuo* (Kugelrohr apparatus (Aldrich), 0.1 mm Hg, T. oven 185-190°C) giving an off-white solid, yield 0.36 g (92%), m.p. 70-71°C. The product gave the following analytical data:

[0070] NMR (CD_3COCD_3, δ, ppm): 3.77, 3.82 (2 × s, 2 × 3H, OMe + OMe), 4.19 (d, 2H, J 5.0 Hz, CH_2OH), 6.25 (dt, 1H, J 5.0 and 15.5 Hz, PhCCH olefinic), 6.51 (d, 1H, J 15.5 Hz, PhCH olefinic), 6.89 (m, 2H, H^{5+6}), 7.05 (br.s., 1H, H^2).

[0071] MS, m/e (rel. intensity, %): 177 (100) [M-OH]+, 195 (4) $[M+H]^+$, 212 (59) $[M+NH_4]^+$, 217 (26) $[M+Na]^+$.

[0072] Example 6: 3,4-Dimethoxycinnamaldehyde (A_7)



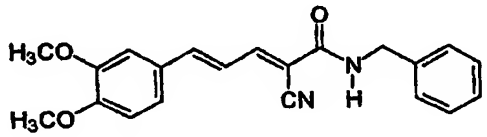
[0073] To a mixture of pyridinium dichromate (3.88 g, 10.3 mmol) and 4 g of finely grounded freshly activated molecular sieves 3Å in 20 ml of CH_2Cl_2 3,4-dimethoxycinnamyl alcohol in 10 ml of CH_2Cl_2 (Example 5, 1.00 g, 5.1 mmol) was added. The reaction was stirred for 2 h, 0.5 ml of methanol was added, the residue was passed through silica gel and washed with 300 ml of ethyl acetate. After evaporation the compound was purified by silica gel chromatography (hexane-EtOAc, 5:1) leading to a crystallizing oil (0.62 g, 63%).

[0074] The product gave the following analytical data:

[0075] NMR (CD_3COCD_3, δ, ppm): 3.90 (2 × s, 2 × 3H, OCH_3 + OCH_3), 6.70 (dd, 1H, J 7.6 and 16.0 Hz, PhC=CH olefinic), 7.05 (d, 1H, J 8.3 Hz, H^5), 7.28 (dd, 1H, J 1.4 and 8.3 Hz, H^6), 7.37 (d, 1H, J 1.4 Hz, H^2), 7.60 (d, 1H, J 16.0 Hz, PhCH olefinic), 9.65 (d, 1H, J 7.6 Hz, CHO).

[0076] MS, m/e (rel. intensity, %): 193 (100) $[M+H]^+$, 210 (26) $[M+NH_4]^+$.

[0077] Example 7: (E,E)-2-(Benzylaminocarbonyl)-3-(3,4-dimethoxystyryl) acrylonitrile (CR2)

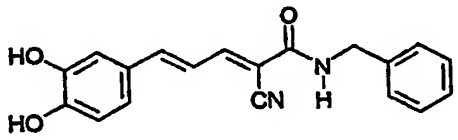


[0078] The compound was prepared as described in Example 3, by adding 3,4-dimethoxycinnamaldehyde (Example 6, 0.04 g, 0.2 mmol) to N-(cyanoacetyl)benzylamide (Example 4, 0.036 g, 0.2 mmol). After refluxing for 1 h and recrystallization from ethanol a yellow solid was obtained (0.045 g, 62%). The product gave the following analytical data:

[0079] NMR (CD_3COCD_3, δ, ppm): 3.90 (s, 2 × 3H, OMe + OMe), 4.57 (d, 2H, J

< 2 Hz, $NHCH_2Ph$), 7.08 (br.s., 1H, H^2), 7.17 (dd, 1H, J 11.5 and 15.2 Hz, PhCCHCCN olefinic), 7.23-7.42 (m, 8H, aromatic + H^5 + H^6 + PhCH olefinic), 7.90 (br.t, 1H, NH), 8.05 (dd, 1H, J 0.55 and 11.5 Hz, CHCN olefinic).

[0080] Example 8: (E,E)-2-(Benzylaminocarbonyl)-3-(3,4-dihydroxystyryl)acrylonitrile (CR4) – Method A

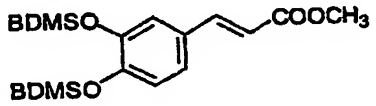


[0081] Boron tribromide (0.033 ml, 0.34 mmol) was added to (E,E)-2-(benzylaminocarbonyl)-3-(3,4-dimethoxystyryl)acrylonitrile (Example 7, 0.04 g, 0.11 mmol). The residue was purified by silica gel chromatography ($CHCl_3$-MeOH, 10:1) to give an orange solid (0.02 g, 55% yield). The product gave the following analytical data:

[0082] NMR (CD_3COCD_3, δ, ppm): 2.86 (br.s., 2H, $(OH)_2$), 4.55 (m, 2H, $NHCH_2Ph$), 6.90-7.42 (m, 10H, Ph + Ph' + olefinic), 7.87 (br.s., 1H, NH), 8.02 (dd, 1H, J < 0.5 and 11.4 Hz, CHCN olefinic).

[0083] MS, m/e (rel. intensity, %): 295 (61) $[M+H-CN]^+$, 321 (100) $[M+H]^+$, 338 (30) $[M+NH_4]^+$.

[0084] Example 9: Methyl ester of 3,4-bis(t-butyldimethylsilyloxy)cinnamic acid (A_8)

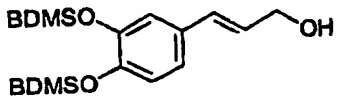


[0085] To a solution of 3.6 g (20 mmol) of 3,4-dihydroxycinnamic acid in 300 ml MeOH was added $SOCl_2$ (100 µl) and the mixture was stirred at 60°C for 5 h. Methanol was taken to dryness and the obtained methyl ester was treated up with 10.2 g (68 mmol) of *t*-$BuMe_2SiCl$ and 9.2 g (136 mmol) of imidazole in 100 ml DMF at

50°C for 0.5 h. Mixture was diluted with water and extracted with hexane. Hexane was taken to dryness. The residue was distilled *in vacuo* (Kugelrohr apparatus (Aldrich), 0.1 mm Hg, T. oven 200-210°C) and crystallized from hexane at -20°C giving a white solid, yield 7.5 g (89%), m.p. 57-58°C. The product gave the following analytical data:

[0086] MS, m/e (rel. intensity, %): 423 (100) $[M+H]^+$, 440 (98) $[M+NH_4]^+$.

[0087] Example 10: 3,4-Bis(t-butyldimethylsilyloxy)cinnamyl alcohol (A_9)

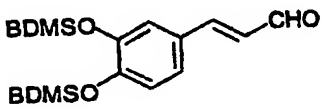


[0088] The compound was prepared as described in Example 5 by treating of 3,4-dihydroxycinnamic acid bis(BDMS) ether methyl ester (Example 9, 0.42 g, 1.0 mmol) with 1M THF solution of diisobutylaluminum hydride (4.0 mmol) in absolute THF (25 ml) at 20°C for 1 h. After distilling *in vacuo* (Kugelrohr apparatus (Aldrich), 0.1 mm Hg, T. oven 185-200°C) a white viscous oil was obtained, yield 0.33 g (85%). The product gave the following analytical data:

[0089] NMR (CD_3COCD_3, δ, ppm): 0.23, 0.24 (2 × s, 2 × 6H, Me_2Si + Me_2Si), 1.00, 1.02 (2 × s, 2 × 9H, *t*-BuSi + *t*-BuSi), 4.19 (d, 2H, J 4.9 Hz, CH_2OH), 6.22 (dt, 1H, J 4.9 and 16.0 Hz, PhCCH olefinic), 6.49 (d, 1H, J 16.0 Hz, PhCH olefinic), 6.85 (d, 1H, J 8.2 Hz, H^5), 6.92 (dd, 1H, J 2.1 and 8.2 Hz, H^6), 6.97 (d, 1H, J 2.1 Hz, H^2).

[0090] MS, m/e (rel. intensity, %): 377 (100) $[M-OH]^+$, 395 (2) $[M+H]^+$, 412 (15) $[M+NH_4]^+$.

[0091] Example 11: 3,4-Bis(t-butyldimethylsilyloxy)cinnamaldehyde (A_{10})



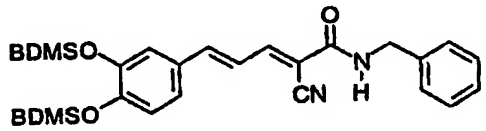
[0092] The compound was prepared as described in Example 6 by adding 3,4-

bis(*t*-butyldimethylsilyloxy)cinnamyl alcohol (Example 10, 0.2 g, 0.5 mmol) in 5 ml of CH_2Cl_2 to a mixture of pyridinium dichromate (0.38 g, 1 mmol) and 1 g molecular sieves 3Å in 20 ml of CH_2Cl_2. The residue was passed through silica gel and washed with 300 ml of EtOAc-hexane, 1:1. After evaporation the compound was purified by silica gel chromatography (hexane-EtOAc, 5:1) leading to an oil (0.15 g, 76%). The product gave the following analytical data:

[0093] NMR (CD_3COCD_3, δ, ppm): 0.26 and 0.28 (2 × s, 2 × 6H, Me_2Si + Me_2Si), 1.01 and 1.02 (2 × s, 2 × 9H, *t*-BuSi + *t*-BuSi), 6.60 (dd, 1H, J 7.7 and 15.9 Hz, PhCCH olefinic), 7.01 (dd, 1H, J < 0.5 and 8.9 Hz, H^6), 7.27 (m, 2H, H^{2+5}), 7.60 (d, 1H, J 15.9 Hz, PhCH olefinic), 9.65 (d, 1H, J 7.7 Hz, CHO).

[0094] MS, m/e (rel. intensity, %): 367 (3) $[M+H-CN]^+$, 393 (100) $[M+H]^+$, 410 (10) $[M+NH_4]^+$.

[0095] Example 12: (E,E)-2-(Benzylaminocarbonyl)-3-(3,4-bis(t-butyldimethylsilyloxystyryl))acrylonitrile (CR18)

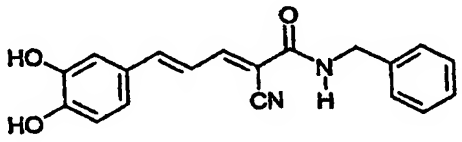


[0096] The compound was prepared as described in Example 3 by adding 3,4-bis(*t*-butyldimethylsilyloxy)cinnamaldehyde (Example 11, 0.100 g, 0.26 mmol) to N-(cyanoacetyl)benzylamide (Example 4, 0.044 g, 0.26 mmol. After refluxing for 2.5 h purification by silica gel chromatography (hexane-EtOAc, 15:1) provided a yellow solid (0.090 g, 64%). The product gave the following analytical data:

[0097] NMR (CD_3COCD_3, δ, ppm): 0.24 and 0.25 (2 × s, 2 × 6H, Me_2Si + Me_2Si), 1.01 and 1.02 (2 × s, 2 × 9H, *t*-BuSi + *t*-BuSi), 4.55 (br.s., 2H, $NHCH_2Ph$), 7.00 (d, 1H, J 8.5 Hz, H^4), 7.12 (dd, 1H, J 11.7 and 15.6 Hz, PhCCHCCN olefinic), 7.24-7.43 (m, 8H, aromatic and olefinic protons), 7.93 (br.s., 1H, NH), 8.02 (dd, 1H, J < 0.5 and 11.7 Hz, CHCN olefinic).

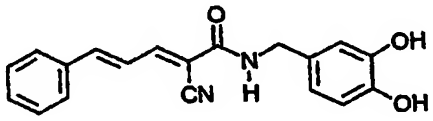
[0098] MS, m/e (rel. intensity, %): 523 (30) $[M+H-CN]^+$, 540 (24) $[M+NH_4-CN]^+$, 549 (89) $[M+H]^+$, 566 (100) $[M+NH_4]^+$.

[0099] Example 13: (E,E)-2-(Benzylaminocarbonyl)-3-(3,4-dihydroxystyryl)acrylonitrile (CR4) – Method B



[00100] (E,E)-2-Benzylaminocarbonyl-3-[3,4-bis(*t*-butyldimethylsilyloxystyryl)] acrylonitrile (Example 12, 0.028 g, 0.052 mmol) was treated with 60 µl of a 1M THF solution of tetra-*n*-butylammonium fluoride in 2 ml of dry THF for 0.5 h at 20°C. After evaporation the compound was dissolved in 5 ml of chloroform-methanol, 20:1, passed through silica gel and washed with chloroform-methanol, 20:1. The residue was purified by HPLC chromatography (MeCN-H_2O, 60:40, UV detection at 340 nm) leading to an orange solid (0.010 g, 62%). The analytical data were identical to the compound prepared as described in Example 8.

[00101] Example 14: (E,E)-2-(3,4 Dihydroxybenzylaminocarbonyl)-3-styrylacrylonitrile (CR19)



[00102] The compound was prepared as described in Example 3 by adding cinnamaldehyde (0.018 ml, 0.14 mmol) to N-(cyanoacetyl)3,4-dihydroxybenzylamide (Example 2, 0.03 g, 0.14 mmol). After refluxing for 2 h and recrystallization from ethanol, a yellow solid was obtained (0.027 g, 59%). The product gave the following analytical data:

[00103] NMR (CD_3COCD_3, δ, ppm): 2.82 (br.s., 2H, $(OH)_2$), 4.39 (br.s., 2H, $NHCH_2Ph$), 6.70 (dd, 1H, J 1.9 and 8.2 Hz, $H^{6'}$), 6.76 (d, 1H, J 8.2 Hz, $H^{5'}$), 6.87 (d,

1H, J 1.9 Hz, $H^{2'}$), 7.30 (dd, 1H, J 11.3 and 15.7 Hz, PhCCHCCN olefinic), 7.47 and 7.73 (2 × m, 6H, aromatic protons and PhCH olefinic), 7.82 (br.s., 1H, NH), 8.04 (dd, 1H, J < 0.5 and 11.3 Hz, CHCN olefinic).

[00104] MS, m/e (rel. intensity, %):321 (100) $[M+H]^+$, 338 (65) $[M+NH_4]^+$.

[00105] Example 15: VEGF secretion from the breast cancer cell lines HTB-133 (KDR+), HTB-131 (KDR-) and MDA-231, HTB-181 prostate cancer cell line, HTB-72 melanoma cell line, CR2-1730 human umbilical vascular endothelial (HUV-EC-C) cell line and normal HUVEC primary cells was measured as follows.

[00106] 10^5-10^6 cells/ml of each of the above cells lines and primary cells were grown for 24 hours in a medium containing 10% FCS which does not contain VEGF. Medium was collected and analysed for the presence of VEGF using VEGF-165 ELISA kit (R&D Systems).

[00107] ***Results:*** All cell lines tested secreted to the medium 2-3 ng/ml of VEGF. No VEGF secretion was detected in normal HUVEC primary cells.

[00108] Example 16: CR-4 dependent inhibition of VEGF secretion from breast, prostate, melanoma and HUVEC cell lines.

[00109] 10^6 cells/ml of each cell lines were incubated for 5 hrs. with various concentrations of CR-4. Cells were washed twice in PBS to remove CR4 and fresh medium was added for 24 hrs. Medium was collected and analysed for the presence of VEGF using VEGF-165 ELISA kit (R&D Systems).

[00110] ***Results:*** As shown in figures 1 to 4, CR-4 dose dependent inhibition, with an IC50 of 10-30 nM, of VEGF secretion was observed in all cell lines.

[00111] Example 17: CR-4 dependent inhibition of VEGF secretion results in the inhibition of HUVEC growth.

[00112] 10^5 cells/ml of KDR^+ HTB-133 and KDR^- HTB-131 breast cancer cell lines were plated and grown over night in HUVEC medium which does not contain VEGF (M199 containing 10% FCS, heparin, antibiotics and endothelial cell growth

supplement). After an incubation for 5 hrs. with various concentrations of CR-4 the wells were washed twice and fresh HUVEC medium was added for 24 hrs. This medium with or without 10 ng/ml of recombinant VEGF was then transferred to parallel wells containing 10^5 cells/ml of HUVEC that were plated and grown in HUVEC medium for 24 hrs. Radioactive labelled [^{3}H]-Thymidine was added to HUVEC wells over night. Cells were harvested and their [^{3}H]-Thymidine incorporation was measured.

[00113] ***Results:*** Figure 5 shows CR-4 dose dependent inhibition, with an IC50 of 20-30 nM, of HUVEC growth is shown. This inhibition is rescued by recombinant VEGF.

[00114] Example 18: CR11 and CR19 dependent inhibition of VEGF secretion from breast cancer cell lines.

[00115] ***Procedure:*** 10^6 cells/ml of each of HTB-133 and MDA-231 cells were incubated for 5 hours with various concentrations of CR11 or CR19. Cells were washed twice in PBS to remove CR11 and CR19 and fresh medium was added for 24 hours. Medium was collected and analysed for the presence of VEGF using VEGF-165 ELISA kit (R&D Systems).

[00116] ***Results:*** Similar to CR-4, the compounds inhibited VEGF secretion in a dose response manner (Figures 6 and 7) and the IC50 values were approximately 30 nM, similar to CR4.

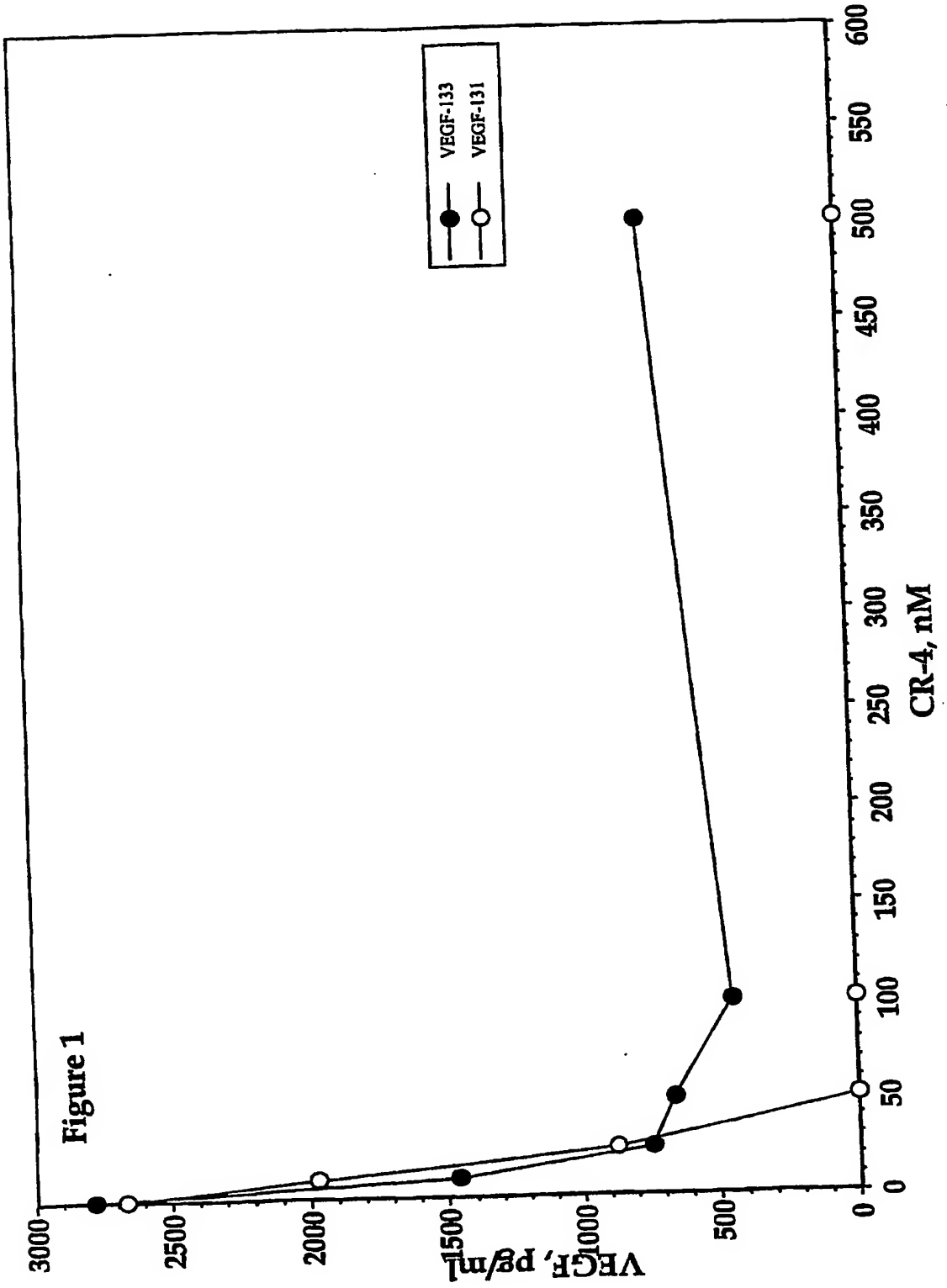
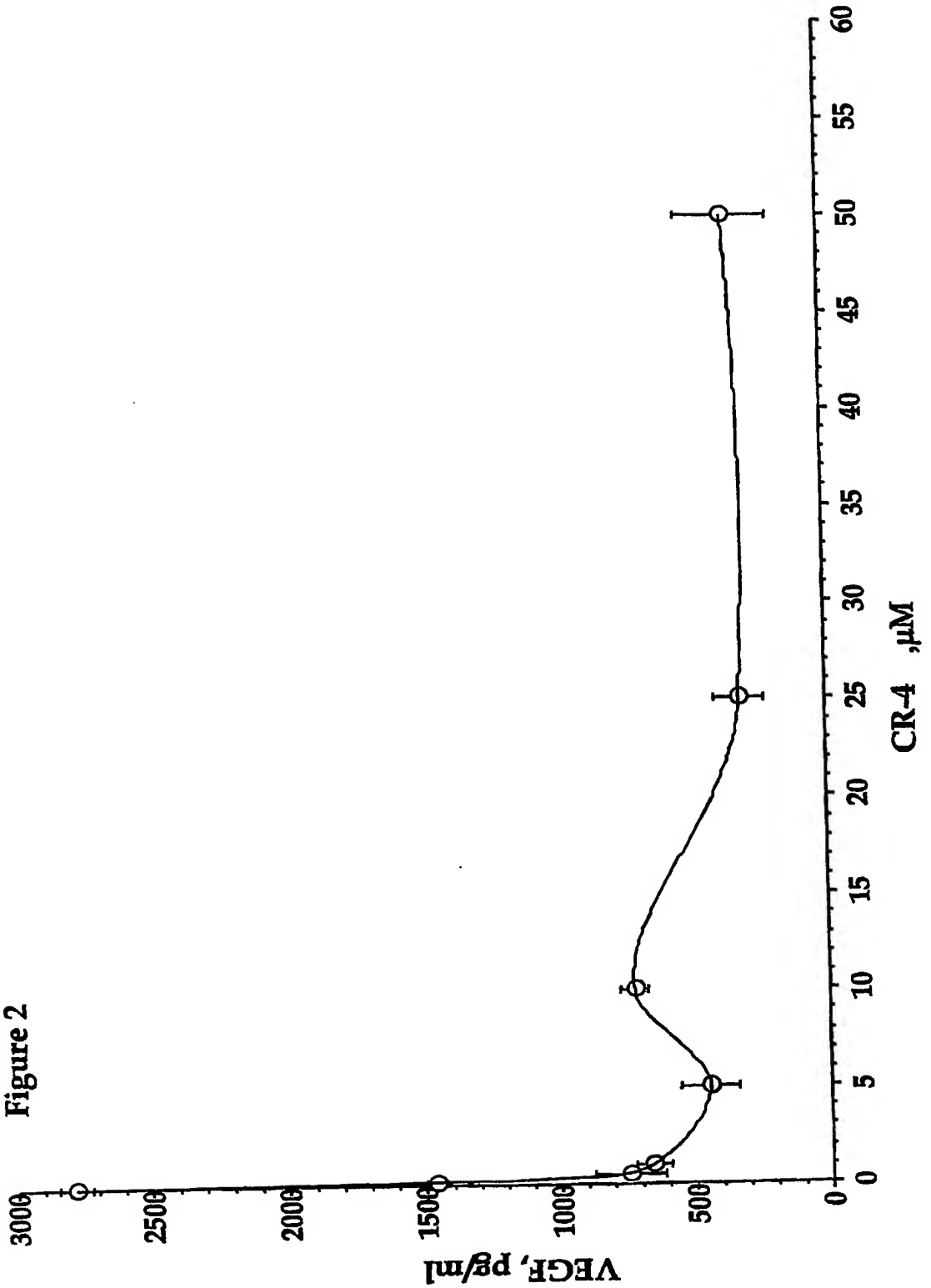

VEGF-133
VEGF-131
VEGF, pg/ml
CR-4, nM
0
500
1000
1500
2000
2500
3000
0
50
100
150
200
250
300
350
400
450
500
550
600

Figure 1

Figure 2


VEGF, pg/ml
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
45
50
55
60
CR-4 ,μM

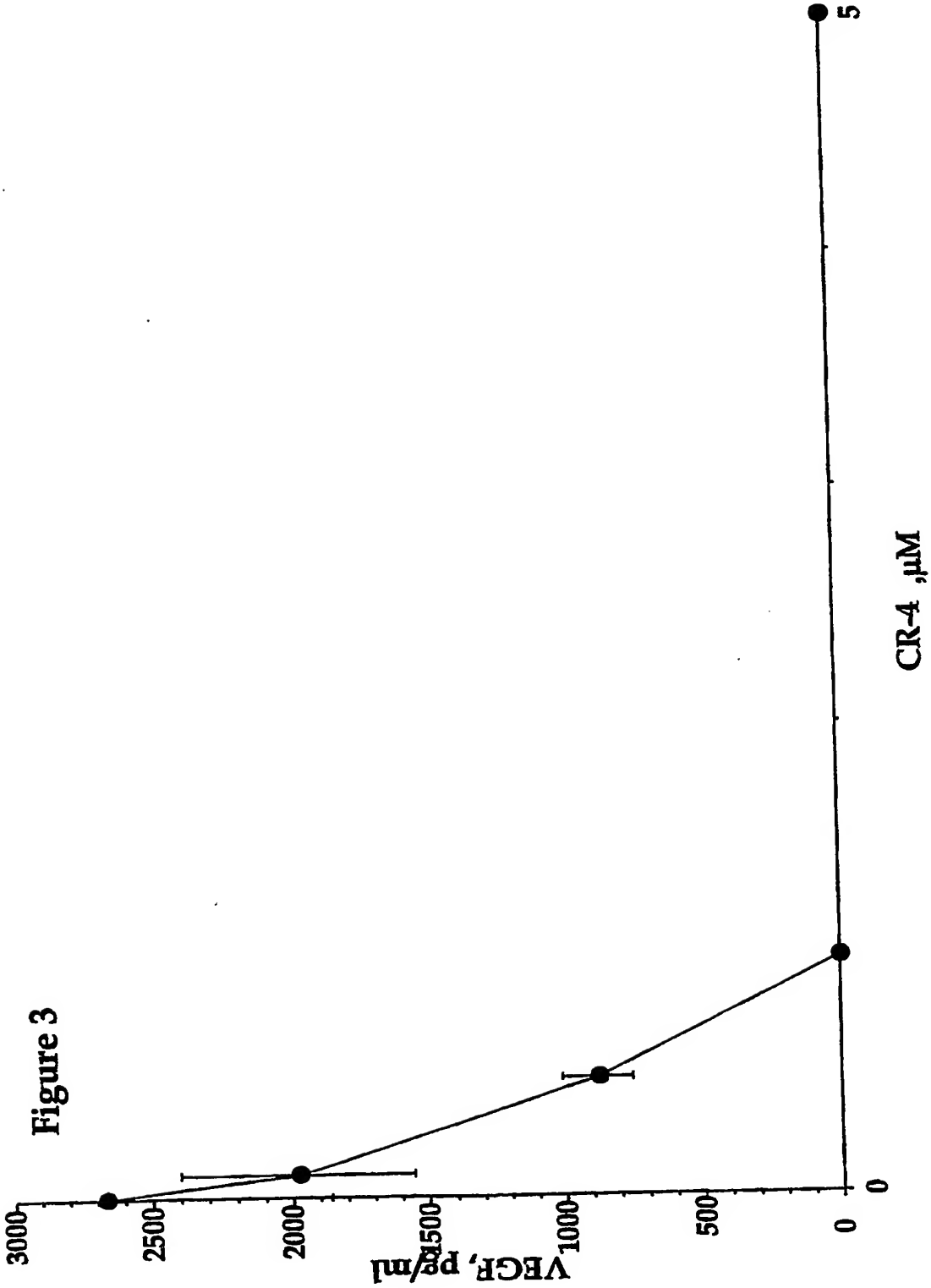


Figure 3

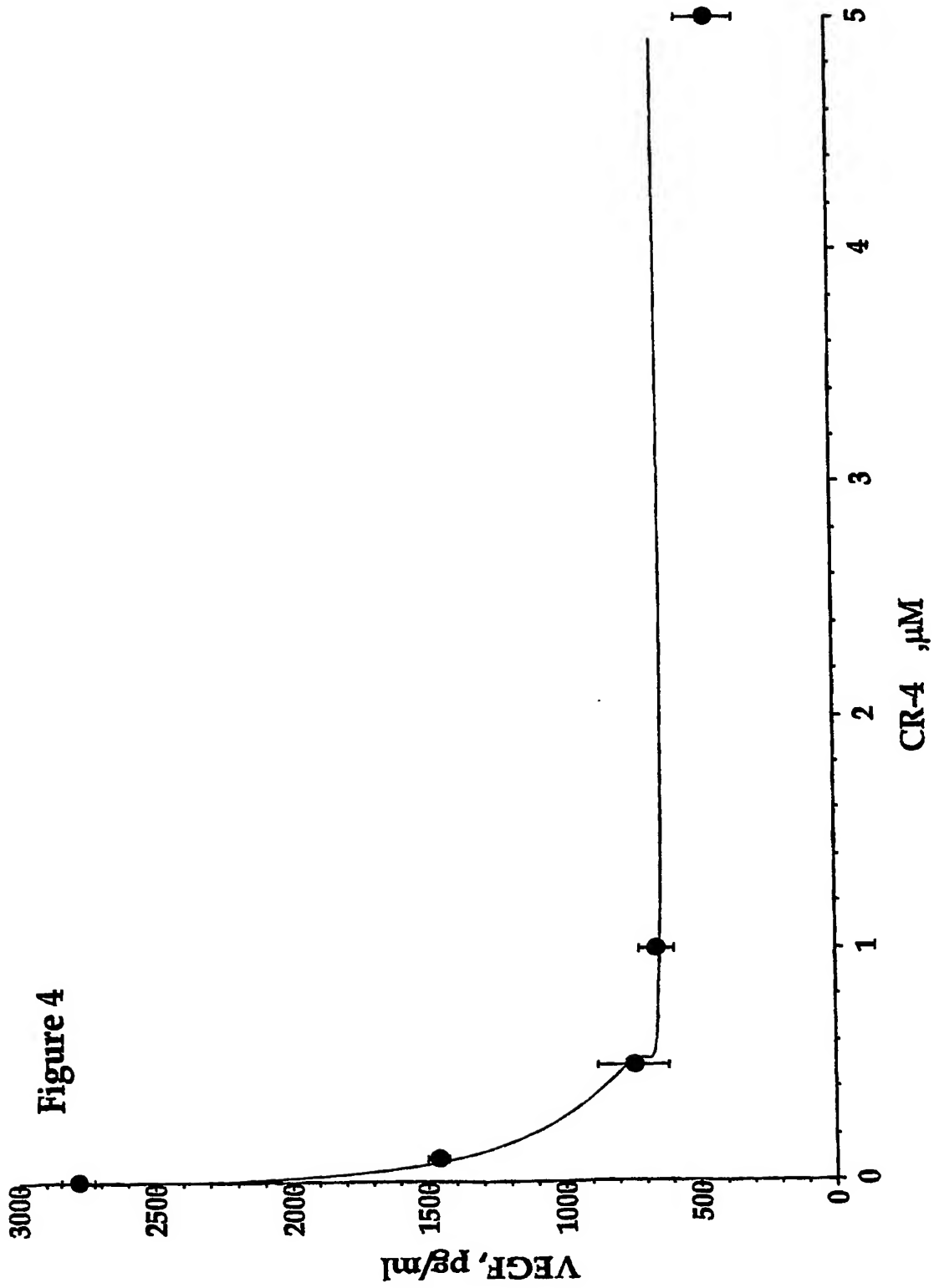


Figure 4

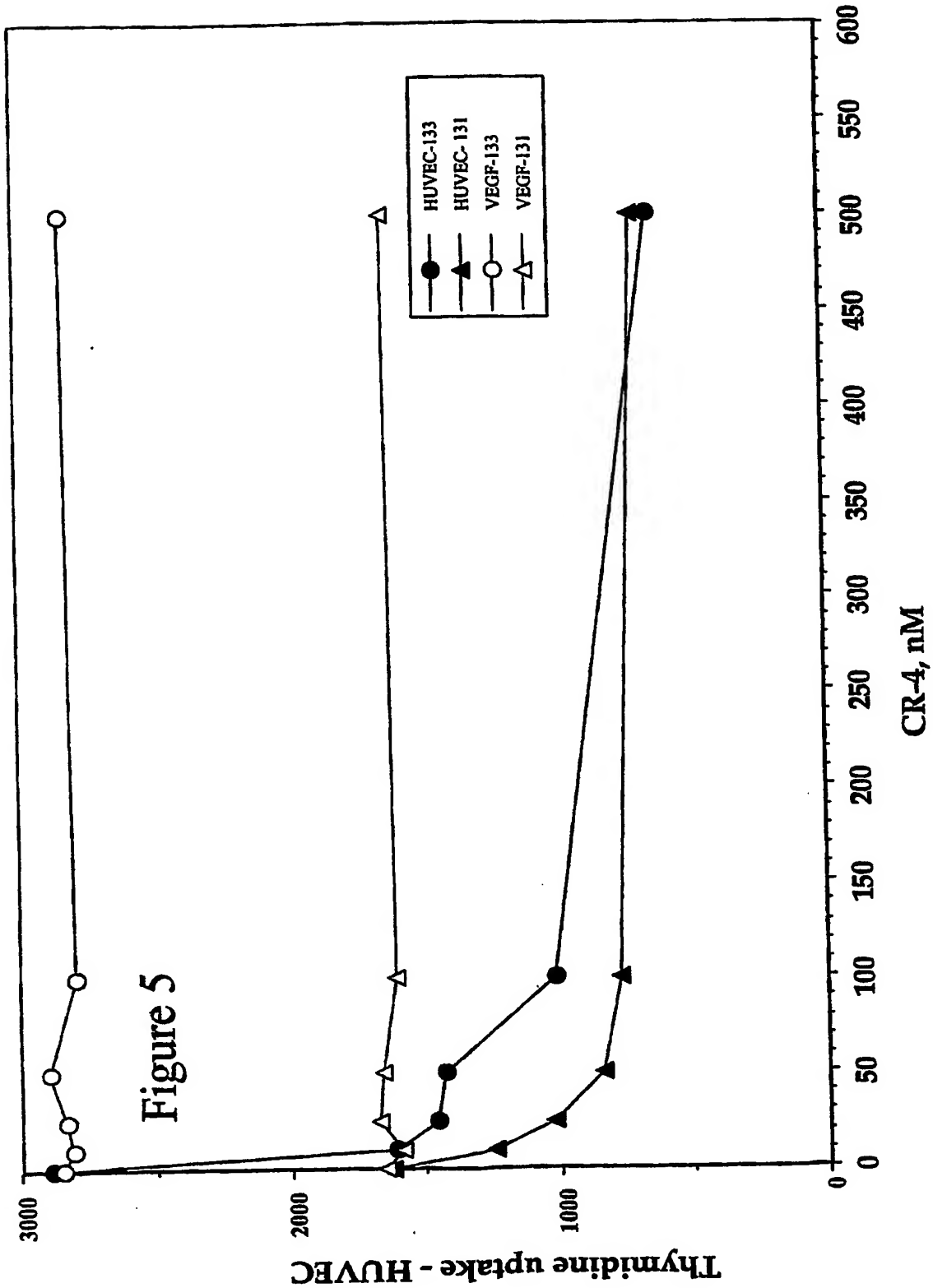

Thymidine uptake - HUVEC
3000
2000
1000
0
0
50
100
150
200
250
300
350
400
450
500
550
600
CR-4, nM
HUVEC-133
HUVEC-131
VEGF-133
VEGF-131

Figure 5

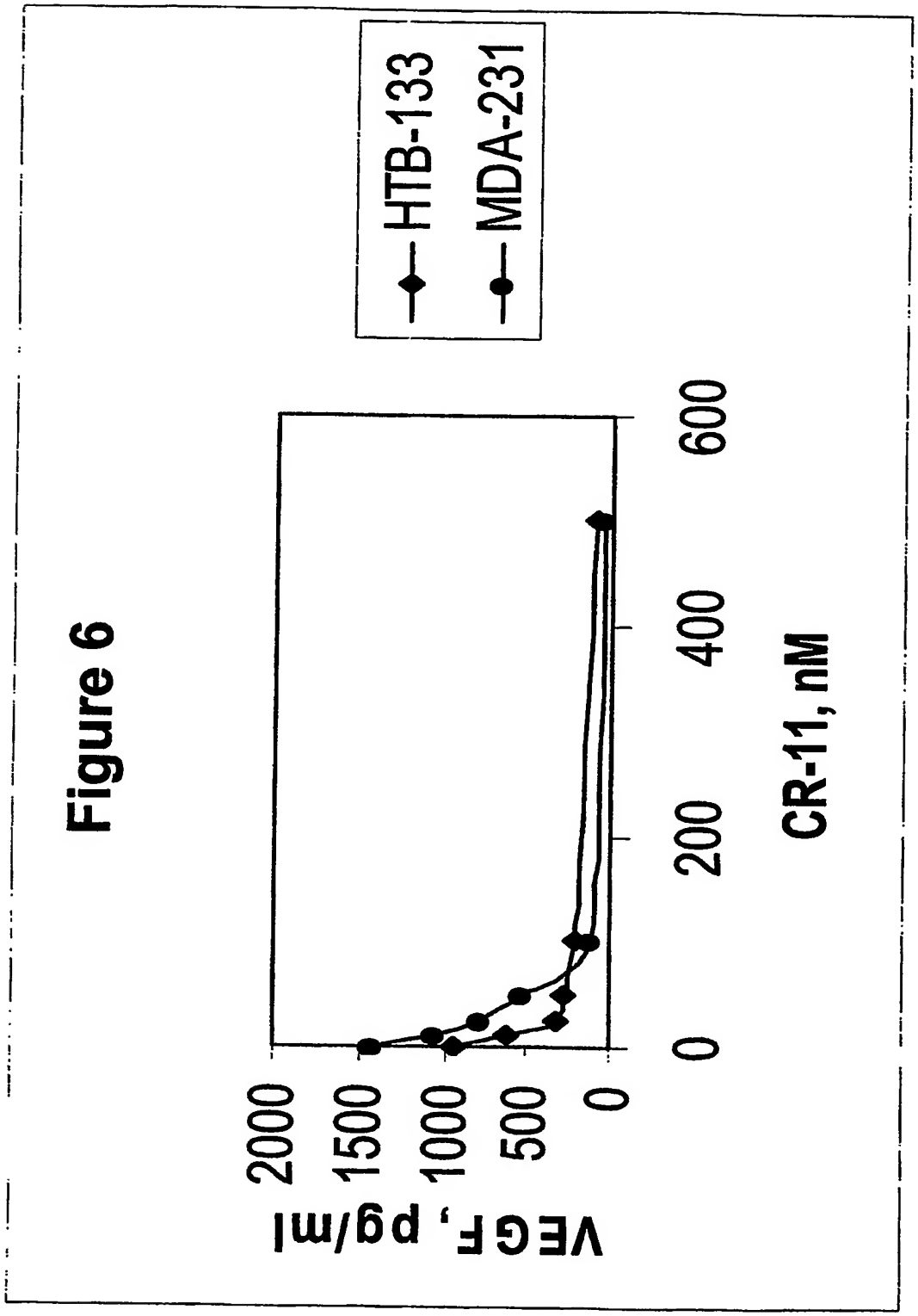

HTB-133
MDA-231
VEGF, pg/ml
2000
1500
1000
500
0
0
200
400
600
CR-11, nM

Figure 6

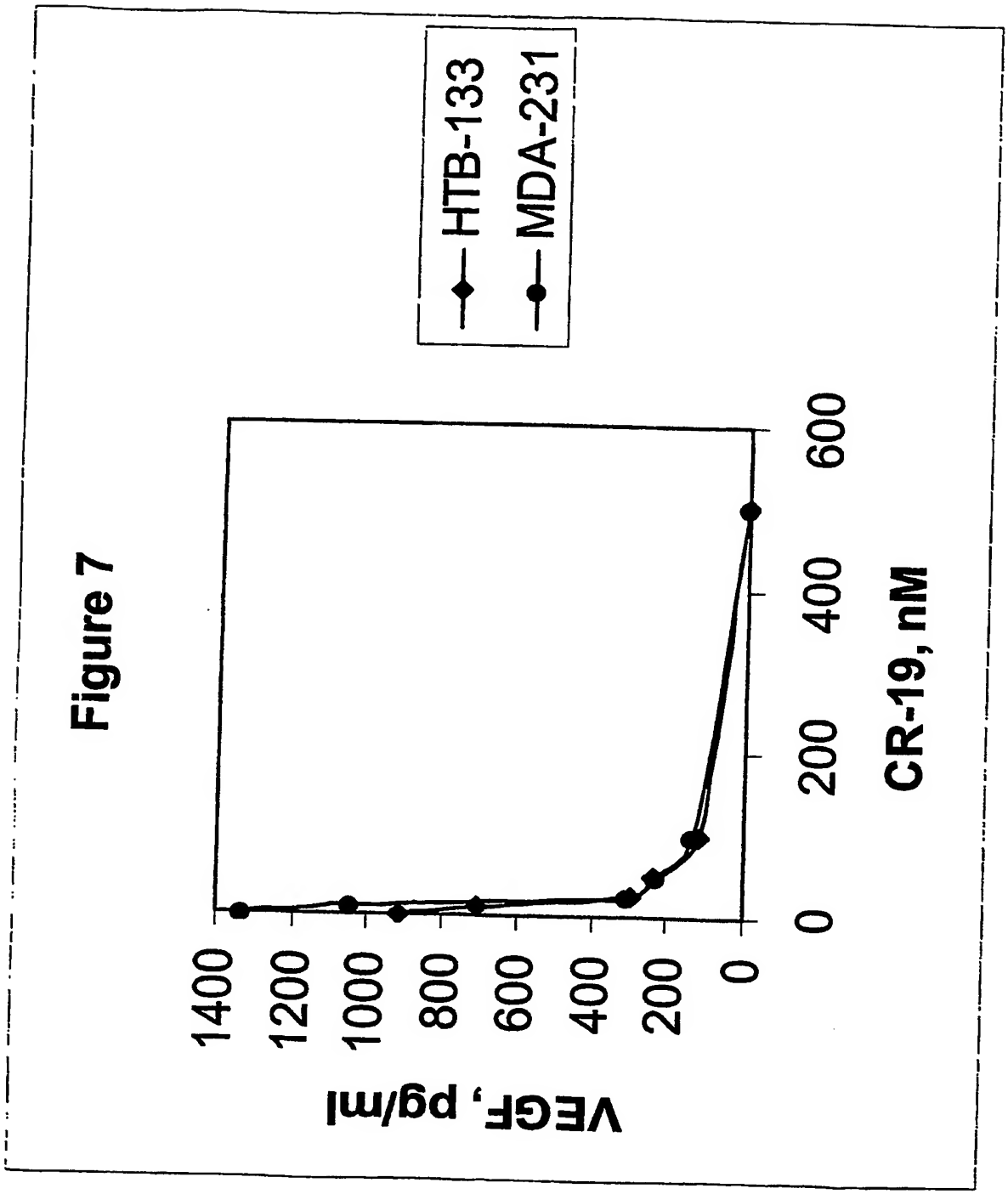

HTB-133
MDA-231
VEGF, pg/ml
1400
1200
1000
800
600
400
200
0
0
200
400
600
CR-19, nM

Figure 7